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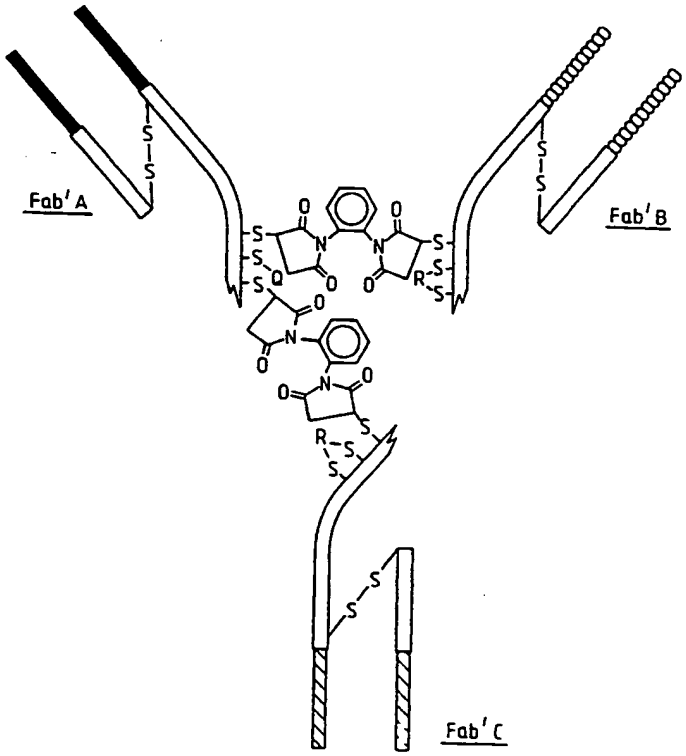
(54) Title: BI-OR TRISPECIFIC $(FAB)_3$ OR $(FAB)_4$ CONJUGATES

(57) Abstract

Novel trimeric and tetrameric antibodies are disclosed, including bispecific and trispecific $F(ab)_3$ and $F(ab)_4$ antibodies. A simple and efficient method is described for the production of pure $F(ab'\gamma)_3$ antibodies, in which the individual antibody Fab' fragments are joined via stable thioether linkages. Hybrid molecules were constructed from mouse monoclonal antibodies with specificities for targetting cytotoxic effectors (human peripheral blood T cells) against ^{51}Cr-labelled chicken red blood cells. Fab' fragments from two of the chosen antibodies were first coupled via their hinge-region SH groups using o-phenylenedimaleimide (oPDM), this bispecific fragment was then linked, again via the hinge region using oPDM, to a third Fab' fragment.



TRISPECIFIC $F(ab')_3$ ABC

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Bi-or trispecific $(Fab)_3$ or $(Fab)_4$ conjugates

Antibodies which are bispecific with respect to the antigen they recognize have been used successfully in a number of applications. In immunochemistry they have been used to cross-link cellular antigen and detecting agent such as ferritin or horseradish peroxidase, doing away with the antibody conjugates used in more conventional methods. Similarly they have been used as heterobifunctional protein cross-linkers for the immobilization of enzymes in a number of assays. Perhaps their greatest potential lies in their therapeutic use for the targeting of unwanted cells or pathogens by cytotoxic effector cells or pharmacologic agents such as drugs or toxins.

It has been demonstrated that bispecific $F(ab'\gamma)_2$ antibodies, in which one $Fab'\gamma$ arm is directed at a lymphoma cell and the other binds to a ribosome-inactivating protein, such as ricin A chain or saporin, can target a toxic agent to tumour cells both in vitro and in vivo and prevent further growth. For targeting effector T cells and polymorphonuclear leukocytes, bispecific antibodies have usually have been employed which cross-link the T cell receptor complex or the Fc receptor, respectively, onto the target cell and thereby mediate high levels of specific lysis. By using the appropriate derivatives in this way, it has been possible to show that both normal circulating T cells and single clones of cytotoxic T lymphocytes can be "armed" to destroy almost any specified target cell, and that lysis is independent of the major histocompatibility complex status of the cells involved. Furthermore, the bispecific antibodies do not simply serve to "glue" the two cell populations together, but in linking the effector and target cell actually trigger the lytic process.

In addition to therapeutic uses, bispecific antibodies have also been useful as tools for understanding some of the molecular

interactions which occur when resting T cells are activated for cytotoxicity and proliferation. At this point the relationship between activation of effectors for lysis and the activation for proliferation is not clear. As a general rule effectors, such as T cells, are not cytotoxic when resting but become cytotoxic when profilterating. However, certain effector populations can be activated to become cytotoxic without being driven into proliferation. While monoclonal antibodies directed at the antigen receptor complex (TCR-CD3) on T cells can, to a limited extent at least, mimic antigen in triggering activation for cytotoxicity and proliferation, it has been shown that bispecific antibodies with dual specificity for the TCR-CD3 and one of a group of accessory T cell molecules, such as CD2 CD4 or CD8, are more potent in this respect.

According to a first aspect of the present invention there is provided a trimeric or tetrameric antibody, preferably bispecific or trispecific. By antibody is meant a moiety capable of binding to one or more specific sites on one or more specific antigens. Trimeric antibodies consist of three structurally similar arms, such as three Fab arms, linked together. Tetrameric antibodies consist of four such arms.

Bispecific antibodies of the invention consist of two arms having a first antigen specificity, the third, and fourth if present, having a second antigen specificity. Trispecific antibodies of the invention consists of three arms having, respectively, first, second and third antigen specificities, if present, the fourth arm has the same antigen specificity as one of the first three arms. Preferred antibodies of the invention are $F(ab)_3$ or $F(ab)_4$ antibodies, such as a bispecific or trispecific $F(ab')_3$ or $F(ab')_4$ antibody and particularly preferably a bispecific or trispecific $F(ab'\gamma)_3$ or $F(ab'\gamma)_4$ antibody. By Fab' antibody is meant an Fab antibody fragment which has been generated by pepsin cleavage of an antibody.

Antibody specificity in antibodies according to the invention may, however, be provided by antibody fragments from any source,

including genetically engineered Fv fragments, which may have engineered on multiple residues suitable for forming links between fragments. Such fragments will form Fv_3 and Fv_4 antibodies.

Antibodies of the invention may have specificities for any antigens against which antibodies can be raised or engineered. They find particular application in therapy, especially against tumour cells, but also have applications in assay techniques.

Preferably, however, at least one arm of the antibody specific for a marker on a target, which may be a target cell such as tumour cell, and at least one arm is specific for a marker on an effector, which may be an effector cell such as a T cell, lymphocyte or macrophage, or it may be another cell toxin such as a ribosome-inactivating protein, for example saporin, ricin A chain or intact ricin, or another therapeutic agent to which antibodies can be raised or engineered, such as daunomycin or adriamycin.

In the case of a trispecific antibody, the trispecificity allows it to at once bind to an effector cell and to activate it. The third arm binds to the target cell. It is preferred that two arms of the trispecific antibody are specific to T cells, one of the CD3 molecule and the other to an accessory surface molecule such as the CD2, CD4 or CD8. Alternatively, both arms may be specific for CD2. In that event, the two arms are specific for different epitopes on CD2 such as $T11_2$ and $T11_3$ or $T11_2$ and $T11_3$.

Also in accordance with the first aspect of the invention there is provided a process for the preparation of a bispecific $F(ab)_3$ antibody comprising:

(i) dissociating a first $F(ab)_2$ antibody fragment having a first specificity into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms;

(iii) linking the first Fab arm from step (i) to the second Fab arm from step (ii), the molar ratio of first Fab arm: a second Fab arm being 2:1, to construct a bispecific $F(ab)_3$ antibody.

Also in accordance with the invention there is provided a process for the preparation of a trispecific $F(ab)_3$ antibody as described above comprising:

(i) dissociating a first $F(ab)_2$ antibody fragment having a first specificity into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms;

(iii) linking the first Fab arm from step (i) to the Fab arm from step (ii) to construct a bispecific $F(ab)_2$ antibody;

(iv) dissociating a third $F(ab)_2$ antibody fragment having a third specificity into its two component Fab arms; and

(v) linking the bispecific $F(ab)_2$ antibody from step (iii) to the Fab arm from step (iv) to give specific $F(ab)_3$.

Preferably, the Fab fragments are generated by treating the antibodies providing the fragments with a proteolytic enzyme such as pepsin to give a monospecific $F(ab)_2$ fragment. This is split by reaction with, for example, 2-mercaptoethanol to give Fab_{SH} fragments, in which the -S-S- links between the Fd chains of the $F(ab)_2$ fragments in the original antibody have been broken and reduced to -SH groups. In the preparation Fab specific antibody, Fab_{SH} fragments are prepared from two antibodies to give $FabA_{SH}$ and $FabB_{SH}$ fragments. The linkage of the Fab_{SH} fragments is effected by treating $FabA_{SH}$ fragments with o-phenylenediamine (oPDM) to give $FabA_{mal}$ fragments. These are combined with untreated $FabB_{SH}$ fragments, in a 2:1 weight ratio, under cross-linking conditions to give bispecific FabAAB. The product was, reduced and alkylated with 20mM 2-mercaptoethanol and 25mM iodoacetamide respectively to remove

any minor products which may have formed by oxidation or disulphide exchange, before fractionating an Ultrogel AcA44. In the preparation of trispecific antibody Fab_{SH} fragments are prepared from three antibodies to give $FabA_{SH}$ $FabB_{SH}$ and $FabC_{SH}$ fragments. The linkage of the Fab_{SH} fragments is effected by treating $FabA_{SH}$ fragments with oPDM to give $FabA_{mal}$ fragments. These are combined with untreated $FabB_{SH}$ fragments under cross-linking conditions to give bispecific $F(ab)_2AB$, as shown in Fig. 1. $FabC_{SH}$ fragments are similarly treated with oPDM, to yield $FabC_{mal}$ fragments, which are combined with the $F(ab)_2AB$ by means of an -SH group on the $F(ab)_2AB$ to give trispecific $F(ab)_3ABC$ antibody (see Fig. 2).

According to a second aspect of the invention there is provided a conjugate comprising an antibody according to the first aspect of the invention and an effector for which at least one of the arms of the antibody is specific. The invention also contemplates a process for the preparation of such a conjugate, in which the antibody is mixed with the effector.

The invention further contemplates a pack comprising an antibody according to the first aspect of the invention and, separately, an effector for which at least one of the arms of the antibody is specific.

The invention will be further described with reference to the example and to the figures, in which:

Figure 1 shows the postulated reaction between two Fab fragments to produce a bispecific $F(ab)_2$ antibody;

Figure 2 shows the proposed structure of a trispecific antibody according to the invention;

Figure 3 shows typical chromatography profiles obtained during the preparation of bispecific $F(ab'\gamma)_3$ and trispecific $F(ab'\gamma)_3$;

Figures 4 (a) and (b) are graphs showing redirected cellular cytotoxicity of ^{51}Cr-labelled chicken red blood cells (CRBC) mediated by normal peripheral blood lymphocyte (PBL) and bispecific $F(ab'\gamma)_2$ antibody;

Figure 5 is a graph showing redirected cellular cytotoxicity of ^{51}Cr-labelled CRBC by PBL and one or two bispecific $F(ab'\gamma)_2$ antibodies;

Figure 6 is a graph showing redirected cellular cytotoxicity of ^{51}Cr-labelled CRBC by bispecific $F(ab'\gamma)_2$ and $F(ab'\gamma)_3$ derivatives;

Figure 7 shows the blocking of redirected cellular cytotoxicity mediated by bispecific $F(ab'\gamma)_2$ and $F(ab'\gamma)_3$ derivatives.

Figure 8 is a graph showing redirected cellular cytotoxicity of ^{51}Cr-labelled CRBC by trispecific $F(ab'\gamma)_3$.

Figures 9(a) and (b) are graphs showing the case of blocking of trispecific antibodies;

Figure 10 is a graph showing proliferation (activation) responses of PBL to different $Fab'\gamma$ derivatives;

Figure 11 shows proliferation responses of PBL to different $Fab'\gamma$ derivatives in the presence and absence of target cells;

Figure 12 shows the redirected cellular cytotoxocity of human tumour cells (Namalwa) with trispecific antibody; and

Figure 13 shows the redirected cellular cytotoxocity of human tumour cells with a trispecific antibody triggering through CD2.

EXAMPLES

Materials:

All cell culture was performed in supplemented DMEM [Dulbecco's Minimum Essential Medium containing glutamine (200 mM), pyruvate (100 mM), penicillin and streptomycin (100 IU/ml), fungizone (2 μg/ml) and 10% FCS (myoclone) (Giboco Ltd, Paisley, Scotland)], or

in supplemented RPMI [RPMI 1640 Medium containing the same supplements as the DMEM, but with the FCS replaced by 10% normal human serum which had been incubated at 56°C for 30 min to destroy any complement-mediated cytotoxic activity].

Antibodies:

A mouse IgG1 monoclonal antibody, $E_{11}C_{12}$, reacting with chicken red blood cells (CRBC) was raised using conventional hybridoma technology. BALB/c mice were immunized in a protocol which delivered CRBC (approx 10^9) s.c. in CFA and IFA (Difco, Detroit, MI) on days 0 and 14 respectively, and i.p. in DMEM on day 24. Four days later splenic mononuclear cells were fused with the NS-1 (P3/NS-1/1-Ag4.1) mouse myeloma line at a ratio of 2:1 by using a standard somatic fusion protocol with polyethylene glycol 4000 (E.Merck, Darmstadt, Germany). Hybridoma cells secreting anti-CRBC antibody were identified by immunofluorescence staining and flow cytometry as described previously and cloned by limiting dilution.

Additional hybridoma cell lines producing the antibodies OKT1 (CD5), OKT3 (CD3) and OKT11 (CD2) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland), and the hybridoma 3G8 (CD16) was a gift from Dr. D. Segal, NIH, Bethesda, Maryland.

All hybridoma cells were expanded as ascitic tumors in pristane-primed (BALB/c x CBA) F1 mice. The 7S IgG fraction of monoclonal ascites was isolated as described by precipitation in 2 M ammonium sulfate, followed by ion exchange chromatography on Trisacryl-M-DEAE (LKB-Produkter AB, Bromma, Sweden).

Antibody $F(ab'\delta)_2$ fragments from IgG were prepared by limited proteolysis with pepsin at pH 4.1-4.2 in 0.1 M sodium acetate. The reaction being monitored at regular intervals by rapid fractionation of 100 µg samples on a GF 250 HPLC column (Zorbac), and then, when less than 10% of the IgG remained, the digestion was stopped by adjusting the pH to 8.0 with saturated Tris-base and the products fractionated on Ultrogel AcA44 (LKB).

Example 1: Preparation of bispecific F(ab'γ)$_3$ antibodies:

F(ab'γ)$_2$ from the two chosen mouse antibodies at 10 mg/ml in 0.2 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA was reduced by addition of 20 mM 2-mercaptoethanol for 30 min at 30°C. At this point both reduced Fab'γ (Fab'γ_{SH}) samples were chilled to 4°C, a temperature which was maintained throughout the remainder of the preparation including the chromatography stages, before running through Sephadex G-25 equilibrated in a buffer of 50 mM sodium acetate, pH 5.3, containing 0.5 mM EDTA. A half volume of 12 mM o-phenylenedimaleimide (oPDM) dissolved in chilled dimethylformamide was then added to one of the two Fab'γ_{SH} samples. After 30 min the maleimidated Fab'γ (Fab'γ_{mal}) was separated from other solutes in the reaction mixture by passage through Sephadex G-25 equilibrated in the 50 mM sodium acetate, pH 5.3, buffer containing 0.5 mM EDTA. It was then added immediately to the Fab'γ_{SH} antibody component of the heterotrimer in a 2:1 molar ratio and concentrated to approximately 5 mg/ml by ultrafiltration under nitrogen using a Diaflo membrane in a chilled Amicon chamber. After incubation for 18 hours, the pH of the reaction mixture was adjusted to 8 using 1 M Tris-HCl, pH 8.0., before reducing with 2-mercaptoethanol at a final concentration of 20 mM for 30 min at 30°C. Finally the bispecific F(ab'γ)$_3$ was separated from other products and residual reactants by passage through Ultrogel AcA44 equilibrated in 0.2 M Tris-HCl. 10 mM EDTA, pH 8.0.

Example 2: Prepartion of trispecific F(ab'γ)$_3$ antibodies:

Firstly, bispecific F(ab'γ)$_2$ antibodies were made by a similar method to that of Example 1, except that the Fab' fragments of Example 1 were mixed in a 1:1 molar ratio, to give bispecific F(ab'γ)$_2$.

Bispecific F(ab'γ)$_2$ was conjugated with a Fab'γ_{mal} from a third antibody. This latter reaction relies on having free -SH groups available in the bispecific F(ab'γ)$_2$ derivative. The procedure and

reaction conditions for linking the Fab'γ_{mal} to the F(ab'γ)$_{2SH}$ were similar to those used Example 1: bispecific F(ab'γ)$_2$ and Fab'γ_2 fragments from the third antibody at 5 mg/ml in 0.2 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA were reduced by addition of 20 mM 2-mercapoethanol for 30 min at 30°C. The samples were chilled to 4°C and run through Sephadex G-25 which had been equilibrated in a buffer of 50 mM sodium acetate, pH 5.3, containing 0.5 mM EDTA. The third Fab'γ_{SH} antibody species was then maleimidated using o-PDM as in the bispecific antibody preparation and finally the bispecific F(ab'γ)$_{2SH}$ and Fab'γ_{mal} were mixed together at a weight ratio of 1:4 for 18 hours at 4°C. Following chormatography on AcA44, 150 kDa-sized material (i.e. F(ab'γ)$_3$) was harvested and concentrated.

Trispecific F(ab'γ)$_4$ was also generated during the trispecific F(ab'γ)$_3$ preparation. It emerged from the AcA44 column at a position which corresponded to that of a protein with a molecular weight of approximately 200 kDa. This size is consistent with the joining of four Fab'γ fragments during the reaction. Apparently, the bispecific F(ab'γ)$_2$ has conjugated with two Fab'γ_{mal} fragments from the third antibody.

The final products in the reaction mixtures were reduced and alkylated with 20 mM 2-mercaptoethanol and 25 mM iodoacetamide respectively to remove any minor products which may have formed by oxidation or disulphide exchange, before fractionating on Ultrogel AcA44.

By these methods, the following antibodies can be prepared:

Bispecific $F(ab)_3$ antibodies

For targeting cytotoxic T cells to unwanted cells:

CD2 x CD2 x target*

CD3 x CD3 x target

CD5 x CD5 x target

CD3 x target x target

Trispecific $F(ab)_3$ antibodies

For targeting cytotoxic T cells to unwanted cells:

CD2 x CD3 x target

CD2 x CD5 x target

CD3 x CD5 x target

CD3 x LFA3 x target

CD3 x CD4 x target

CD3 x CD8 x target

$CD2(T11_1)$ x $CD2'(T11_3)$ x target

* target = CRBC or a tumor specific antigen

For targeting cytotoxic agents to unwanted cells:

toxin ** x Ig x CD19

toxin x Ig x CD22

toxin x Ig x CD37

** toxin = ribosome inactivating proteins such as saporin or ricin

The proposed structure of a trispecific $F(ab'\gamma)_3$ antibody of the invention specific for antigens A, B and C is shown in Fig. 2. Although not shown, some of the γ-L chain disulfide bonds will be reduced during the preparation. Such reduction is known not to compromise antigen binding activity in Fab fragments. Before the final product was alkylated with iodoacetamide, one hinge-region sulphydryl (-SH) group remained, offering the potential for linking at least one more $Fab'\gamma_{mal}$ fragment yielding $F(ab'\gamma)_4$, as mentioned above. The groups joined to the cysteinyl sulfur are: Q is carboxyamidomethyl 2, a blocking group; and R is o-phenylenedisuccimimidyl.

Typical chromatography profiles obtained during the preparation of (a) bispecific $F(ab'\gamma)_3$ and (b) trispecific $F(ab'\gamma)_3$ derivatives are shown in Fig. 3 (a) and (b) respectively

(a) A reaction mixture containing Fab'_{mal} and Fab'_{SH} at a weight ratio of 2:1 was reduced and alkylated and then fractionated on Ultrogel AcA44 in 0.2 M Tris HCl, pH 8.0. The unreacted Fab' fragments and the bispecific $F(ab'\gamma)_2$ and $F(ab'\gamma)_3$ are indicated.

(b) A reaction mixture containing bispecific $F(ab'\gamma)_{2\ SH}$ and Fab'_{mal} at a weight ratio of 1:4 was reduced and alkylated and then fractionated as for (a). $Fab'\gamma$, $F(ab'\gamma)_2$, $F(ab'\gamma)_3$ and putative $F(ab'\gamma)_4$ are indicated.

Target cells:

Chicken red blood cells (CRBC) and Namalwa lymphoma cells were used as targets throughout the study. As regards the CRBC, fresh blood was collected from Rhode Island Red Chickens into preservative-free heparin and washed in DMEM before storing in supplemented DMEM at 4°C. For radiolabelling, 50µl of CRBC ($5x10^8$) or 200µl of Namalwa ($2 x 10^7$) were first incubated in 250 µl $Na_2{}^{51}CrO_4$ (Amersham International UK) for 40 min at 37°C and then washed four times in DMEM before resuspending at $2x10^5$/ml in supplemented DMEM.

Effector Cells:

Donors for PBL were healthy laboratory personnel in the age range of 21 to 55 years. Blood was collected into preservative-free heparin and separated by flotation on Ficoll-Hypaque (Lymphoprep, Nyeguard, Oslo, Norway). Cells collected at the interface were washed in phosphate-buffered saline (PBS) and resuspended in supplemented DMEM for use in cytotoxicity or proliferation assays.

Redirected cellular cytotoxicity (RCC) assay:

Cytotoxicity was measured by a standard ^{51}Cr-release assay in 96-well, U-bottomed, microculture plates (Gibco). Each well received 50 µl of antibody diluted in supplemented DMEM, followed by 10^4 5^1Cr-labeled CRBC (50 µl) or 10^4 ^{51}Cr-labeled Namalwa cells (50 µl) and $2x10^5$ or $5x10^5$ PBL effectors (100 µl) respectively in supplemented DMEM. The cell mixtures were then sedimented by centrifugation (230xG for five min) before incubating at 37°C in a humidified atmosphere of 5% CO_2 in air for 4, 8 or 21 hours to allow lysis. Finally the cells were sedimented at 420xG for five min and 100-µl aliquots of supernatant removed to assess the release of ^{51}Cr from target cells. Percentages of specific ^{51}Cr release were calculated by the usual method using detergent lysis with 1% Nonidet P40 to give maximum ^{51}Cr release.

Graphs showing redirected antibody dependent cellular cytotoxicity of ^{51}Cr-labeled CRBC mediated by normal PBL and bispecific $F(ab')_2$ antibody are shown in Fig. 4:

(a) shows lytic activity of the CD3 x CRBC antibody (100 ng/ml) in 4 and 8 hour assays using fresh PBL from six healthy donors.

(b) shows lysis of CRBC using PBL from one healthy donor and various concentrations (as indicated) of CD3 x CRBC and CD16 x CRBC in 4, 8 or 21 hour assays. The assay time is indicated on each of the CD3 x CRBC titration curves, but is omitted from those of the CD16 x CRBC derivative due to their proximity.

Note that while there is considerable variation between donors, all have shown appreciably higher levels of lysis in the longer assay. With two of the donors it was only in this longer 8 hour assay that significant release of ^{51}Cr could be measured at all. This result is confirmed and extended for one donor in Figure 4b, which shows that near maximal lytic activity was approached in eight hours with an antibody concentration of 100 ng/ml.

A graph showing redirected antibody dependent cellular cytotoxicity of ^{51}Cr-labeled CRBC by PBL and one or more bispecific $F(ab'\gamma)_2$ antibodies is shown in Fig. 5. Lysis was measured in an 8 hour assay using fresh PBL from one donor and the derivatives indicated.

Results indicated that while the CD3 x CRBC $F(ab'\gamma)_2$ lysed CRBC at concentrations as low as 4 ng/ml, almost 1000 times more CD2 x CRBC $F(ab')_2$ was necessary to achieve appreciable levels of cytotoxicity. Mixing the CD2 bispecific reagents with the CD3 x CRBC antibody resulted in no additive effects, giving similar levels of killing to those seen with the CD3 derivative alone.

Fig. 6 shows that the redirected cellular cytotoxicity against ^{51}Cr-labelled CRBC is considerably higher for bispecific $F(ab'\gamma)_3$ derivatives than for bispecific $F(ab'\gamma)_2$ derivatives. For example, the CD3 x CD3 x CRBC trimer was found to be up to 125 times more potent than the equivalent dimer, CD3 x CRBC, giving significant activity at concentrations below 0.1ng/ml.

The efficiency of these bispecific trimers is not dependant upon two Fab'δ arms being bound to the effector cells, since the derivative CD3 x CRBC x CRBC gives a similar cytotoxicity result. The increased efficiency probably results from the increased binding avidity of the bispecific trimers. Blocking studies with excess Fab'δ (Fig. 7) showed that the redirected cellular cytotoxicity mediated by a trispecific trimer is far more readily inhibited by blocking with the appropriate Fab'δ antibody than is that of a bispecific trimer.

Fig. 7a shows a comparison of the cytotoxicity of CD2 x CRBC antibody with that of CD2 x CD2 x CRBC antibody, blocked with CD2 antibody. Fig. 7b shows a comparison of the cytotoxicity of CD3 x CRBC antibody with that of CD3 x CD3 x CRBC antibody, blocked with CD3 antibody.

These graphs show that redirected cellular cytotoxicity mediated by bispecific dimers is completely inhibited by the addition of 25μg/ml of the appropriate Fab'δ, whilst under similar conditions bispecific trimers still showed cytotoxic activity with blocking Fab'δ at 500μg/ml.

A graph showing redirected antibody dependent cellular cytotoxicity of ^{51}Cr-labelled CRBC by trispecific $F(ab'\delta)_3$ is shown in Fig. 8. Lysis was measured in an 8 hour cytotoxicity assay using fresh PBL from one donor and the derivatives indicated.

The results show that a trispecific $F(ab'\delta)_3$ derivative containing anti-CD2 and -CD3 antibody specificities was at least 100 times more potent than the best bispecific $F(ab'\delta)_2$ derivative and could promote significant levels of lysis at concentrations well below 0.1 ng/ml.

From these results, it appears that trispecific $F(ab)_3$ antibodies according to the invention when used therapeutically will be effective at doses as low as 1% of those required for similar bispecific $F(ab)_2$ antibodies. This has clear advantages both in

cost of treatment and directly to the patient who will take a lower dosage of antibody.

To confirm that both the arms of the trispecific antibody specific for the effector were required for full activity we performed RCC assays in the presence of blocking Fab' δ antibody. Figure 9(a), shows the cytotoxicity of the CD2 x CD3 x CRBC antibody in the presence of, respectively, CD2 antibody, CD3 antibody and a mixture of CD2 antibody and CD3 antibody as blocker. The graph shows that when using CD2 antibody CD3 antibody at 500 μg/ml, a concentration known to be sufficient to block bispecific derivatives (Fig. 7) no reduction in the activity of this trispecific reagent was observed. It was only when both these blocking antibodies were included in the assay that any reduction in activity occurred. With Fab' from both CD2 antibody and CD3 antibody each 500 μg/ml, specific ^{51}Cr release was reduced from 65% to 15%. In a similar experiment the results of which appear in Fig. 9(b), the trispecific $F(ab'\gamma)_3$ antibody CD3 x CD5 x CRBC also demonstrated redirected cellular cytotoxicity which was much more resistant to blocking than a bispecific $F(ab'\gamma)_2$ (See Fig. 7). In this example however, the blocking was slightly more effective than that with the CD2 x CD3 x CRBC reagent. While this increased sensitivity to blocking could reflect a reduced avidity by the CD3 x CD5 x CRBC antibody, it is probably also affected by the relatively poor cytotoxic potency of this reagent.

Incorporation of [^{3}H]thymidine:

Proliferation of normal T cells in response to mitogenic antibody derivatives was assessed in vitro. Peripheral blood lymphocytes (PBL) isolated from Ficoll-Hypaque were cultured at 37°C in 96-well, U-bottomed, microculture plates (10^5/well) in supplemented RPMI containing the various antibody derivatives, together with or without CRBC (200 μl /well). After 48 hours each well was pulsed for 16 hours at 37°C with 1 μCi [^{3}H]thymidine (Amersham) and the incorporated radioactivity harvested onto glass microfibre filters and assessed as described previously. All experimental points were determined in triplicate.

A graph showing proliferation (activation) responses of PBL to different Fab'γ derivatives is shown in Figs. 10 and 11. Fresh PBL from one donor were cultured for 48 hours with the Fab'γ derivative or mixture of derivatives indicated.

The results show that in the absence of CRBC only the trispecific antibody, CD2 x CD3 x CRBC, and to a lesser extent CD3 x CD5 x CRBC, gave a significant proliferation signal. In contrast when CRBC were included in the cultures a number of antibodies, particularly CD2 x CD2 x CRBC and a mixture of CD2 x CRBC + CD3 x CRBC were highly mitogenic.

Bispecific or trispecific $F(ab)_3$ antibodies with the appropriate specificities can be constructed which will first activate cellular effectors, such as T cell or monocytes, and then target them to destroy any unwanted cell. In the case of trispecific antibodies, efficient activation would appear to benefit from two antibody specificities reacting with molecules on the surface of the effector cell. The third Fab specificity of the trispecific $F(ab)_3$ is then available to target against any unwanted cell. Target cells in this system could include neoplastic cells, virally infected host cells (including HIV), autoreactive host cells (B or T lymphocytes) or invading pathogens, including bacteria and viruses.

The bispecific $F(ab'\gamma)_3$ antibodies approximately 100 times more potent than the equivalent bispecific $F(ab'\gamma)_2$ antibodies. For example, CD3 x CD3 x CRBC antibody was still fully active at below 1ng/ml. This surprising increase in performance does not appear to arise from more efficient triggering of cytotoxic T cells, at least as judged by triggering of mitosis. An $F(ab'\gamma)_3$ antibody containing two CRBC specific Fab'γ arms and one T cell specific Fab'γ arm also demonstrated a similar improvement in performance. It seems likely that bispecific $F(ab'\gamma)_3$ antibodies, because they bind to one cell surface through two Fab'γ arms, couple target and effector cells together with an increased avidity over equivalent bispecific $F(ab'\gamma)_2$ antibody. Blocking studies were consistent with this interpretation, showing that the two arms of a bispecific $F(ab'\gamma)_3$

required at least 20 times more free Fab'δ from the appropriate antibody to inhibit cytotoxicity than did equivalent $F(ab'\delta)_2$ reagent.

In the case of trispecific antibodies, it may be advantageous to have two antibody Fab arms reacting with the target cell and one Fab arm recruiting either a cellular effector or a pharmacological agent such as a toxin. As with the effector T cell, other cells, including B cells and monocytes, can be activated when bound by two antibodies reacting with the appropriate surface molecules. Activiated cells which show a high proliferative rate are also often more susceptible to destruction by pharmacological agents such as cytotoxic drugs and toxins; the more rapidly growing tumours, such as childhood acute lymphoblastic leukaemia, are often the most sensitive to conventional chemotherapy. Thus, a trispecific antibody, with two Fab arms directed at the target cell and one at a pharmacological agent, could first activate the target cell and then deliver a poison while it remained in a hypersensitive state.

In addition to the advantages of being able to activate effector or target cells, trispecific antibodies, because they have two Fab arms binding to one surface, also display an increased avidity for that surface. A trispecific $F(ab)_3$ derivative will cross-link two cell surfaces together significantly more strongly than a mixture of two bispecific $F(ab)_2$ antibodies. This advantage may also be applied to immunoassays including enzyme-linked immunosorbent assays or radio-immunoassays. In this situation a trispecific antibody with two different binding sites for a single antigen, such as an enzyme, protein or peptide, and a third Fab arm for a second protein, can be used as a single step cross-linker increased avidity.

Other immunoassays in which a trispecific $F(ab)_3$ derivatives may be useful include situations where it is necessary to capture three different immunogenic antigens, such as enzymes, proteins or peptides, into a tight immune complex. An enzyme and its substrate could be captured directly from solution onto a solid surface in this way.

Redirected cellular cytotoxicity (RCC) of human tumour cells (Namalwa) with trispecific antibody.

Fresh human blood mononuclear cells (PBL) and ^{51}Cr-labelled Namalwa cells were mixed in a standard 20 h cytotoxicity assay with derivatives of the shown specificities. The differentiation antigen CD37 was used as a tumour marker for delivering effectors to the Namalwa cells. The results are shown in Figure 12 of four RCC assays, using PBL from different donors (donor 1-4). In each case the trispecific antibody [anti-CD2 x anti-CD3 x anti-CD37] is far more active than any bispecific antibody. Note that this enhance lysis is specific in that the derivative [anti-CD2 x anti-CD3 x anti-CRBC], which is mitogenic to T cells and was highly potent in RCC against CRBC (Fig. 8), showed no cytotoxic activity (Donor 2). Also a mixture of two bispecific antibodies, [anti-CD3 x anti-CD37] + [anti-CD2 x anti-CD37], was no more active than [anti-CD3 x anti-CD37] alone (Donor 3). These results confirm the CRBC results by showing that a derivative with a pair of antibody arms (anti-CD2 x anti-CD3) which activate cytotoxic T cells, become highly potent reagents for killing unwanted targets when converted into a trispecific antibody by the addition of an anti-target cell arm.

RCC of human tumour cells with a trispecific antibody triggering through CD2.

The experimental conditions were the same as those used in Figure 12, but using effectors from two different donors and derivatives with the specificities shown in Figure 13. The results in Figure 13 show that, for both donors a trispecific antibody derivative containing a mitogenic pair of anti-CD2 antibodies, anti-CD2 ($T11_1$) and anti-CD2' ($T11_3$), is as, or more, active than the original trispecific antibody [anti-CD2 x anti-CD3 x anti-CD37]. Note that three other trimeric antibodies, [anti-CD3 x anti-CD3 anti-CD37], [anti-CD3 x anti-CD4 x anti-CD37] and [anti-CD3 x anti-CD5 x anti-CD37], which are not mitogenic to T cells do not show the enhance activity of the [CD2 x CD3 x CD37] and [CD2 x CD2' xCD37] derivatives, but are probably more active than a bispecific $F(ab')_2$ [anti-CD3 x anti-CD37].

Claims

1. A trimeric or tetrameric antibody.

2. A bispecific or trispecific antibody according to claim 1.

3. An antibody according to any preceding claim comprising at least one arm specific for a marker on a first moiety and at least one arm specific for a marker on a second moiety.

4. A trispecific antibody according to any preceding claim comprising one arm specific for a marker on a first moiety and two arms specific, respectively, for different markers on a second moiety.

5. An antibody according to claim 3 or 4 in which the first and second moieties are, respectively, a target cell and an effector.

6. An antibody according to claims 3 or 4 in which the first and second moieties are, respectively, an effector and a target cell.

7. A antibody according to claim 5 or 6 in which the effector is an effector cell.

8. An antibody according to claims 5 or 6 in which the effector is a therapeutic agent effective to destroy the target cell.

9. An antibody according to claim 8, in which the therapeutic agent is a conventional chemotherapeutic compound to which antibodies can be raised, such as daunomycin or adriamycin.

10. An antibody according to any of claims 5 to 9 in which the target cell is a tumour cell.

11. A trispecific antibody according to any of claims 1 to 5, in which two arms are specific for, respectively, TCR-CD3 and an accessory molecule on effector T cells.

12. A trispecific antibody according to any of claims 1 to 5 comprising two arms specific for, respectively, different sites on a Type 1 or Type 2 ribosome-inactivating protein and at least one arm specific for a marker on a target cell.

13. A trispecific antibody according to any of claims 1 to 4, 6 and 8 to 10 comprising two arms specific for, respectively, two different markers on a target cell and at least one arm specific for a Type 1 or Type 2 ribosome-inactivating protein.

14. A trispecific antibody according to claim 12 or 13, in which the ribosome-inactivating protein is saporin, ricin A chain or intact ricin.

15. A bispecific antibody according to any of claims 1 to 3, 5 to 7 and 10 comprising two arms specific for TCR-CD3.

16. A bispecific antibody according to any of claims 1 to 3, and 5 comprising two arms specific for a target cell.

17. An antibody according to any preceding claim in which the monomer arms are linked by a -S-(o-phenylenedisuccinimidyl)-S- group.

18. A $F(ab)_3$ or $F(ab)_4$ antibody according to any preceding claim.

19. An $F(ab\gamma)_3$ or $F(ab\delta)_4$ antibody according to any preceding claim.

20. An $F(ab')_3$ or $F(ab')_4$ antibody according to any preceding claim.

21. An $F(ab'\gamma)_3$ or $F(ab'\gamma)_4$ antibody according to any preceding claim.

22. A Fv_3 or Fv_4 antibody according to any of claims 1 to 17.

23. A process for the preparation of a bispecific $F(ab)_3$ antibody comprising:

(i) dissociating an $F(ab)_2$ antibody fragment having a first specificty into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms; and

(iii) linking the first Fab arm from step (i) to two component Fab arms from step (ii) to give bispecific $F(ab)_3$.

24. A process for the preparation of a trispecific $F(ab)_3$ antibody comprising:

(i) dissociating an $F(ab)_2$ antibody fragment having a first specificity into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms;

(iii) linking the first Fab arm from step (i) to the Fab arm from step (ii) to construct a bispecific $F(ab)_2$ antibody;

(iv) dissociating a third $F(ab)_2$ antibody fragment having a third specificity into its two component Fab arms; and

(v) linking the bispecific $F(ab)_2$ antibody from step (iii) to the Fab arm from step (iv) to give trispecific $F(ab)_3$.

25. A process according to claim 23 or 24 in which the $F(ab)_2$ antibody fragments used in steps (i), (ii) and (iv) are obtained by treating antibodies having the said specificities with pepsin or other proteolytic enzyme.

26. A process according to any of claims 23 to 25 in which one or more of steps (i), (ii) and (iv) are carried out by treatment of the $F(ab)_2$ antibody fragment with a reducing agent such as 2-mercaptoethanol.

27. A process according to any of claims 23 to 26 in which the linking of step (iii) is effected by treating the dissociated Fab fragment produced in one of steps (i) and (ii) with o-phenylenedimaleimide and combining the thus treated Fab fragment with the untreated Fab fragment produced in the other of steps (i) and (ii) under cross-linking conditions to give the bispecific $F(ab)_2$ fragment.

28. A process according to any of claims 24 to 27 for the reporting of a trispecific $F(ab)_3$ antibody in which the linking of step (v) is effected by treating the Fab fragment produced in step (iv) with o-phenyleledimaleimide and combining the thus treated Fab fragment with the bispecific $F(ab)_2$ fragment produced in step (iii) under cross linking conditions to give $F(ab)_3$.

29. A conjugate comprising a bispecific or trispecific antibody according to any of claims 1 to 22 or produced by a process according to any of claims 23 to 28 complexed with an effector to which at least one arm of the antibody is specific.

30. A process for the preparation of a conjugate according to claim 29, comprising mixing the antibody with the effector.

31. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, either: a bispecific or trispecific antibody according to any of claims 1 to 22; a bispecific or trispecific antibody produced by a process

according to any of claims 23 to 28; or a conjugate according to claim 29, or a conjugate produced by a process according to claim 30.

32. A pack comprising: a bispecific or trispecific antibody according to any of claim 1 to 22 or produced by a process according to any one of claims 23 to 28 and, separately therefrom, an effector for which at least one arm of the antibody is specific.

Fig. 1.

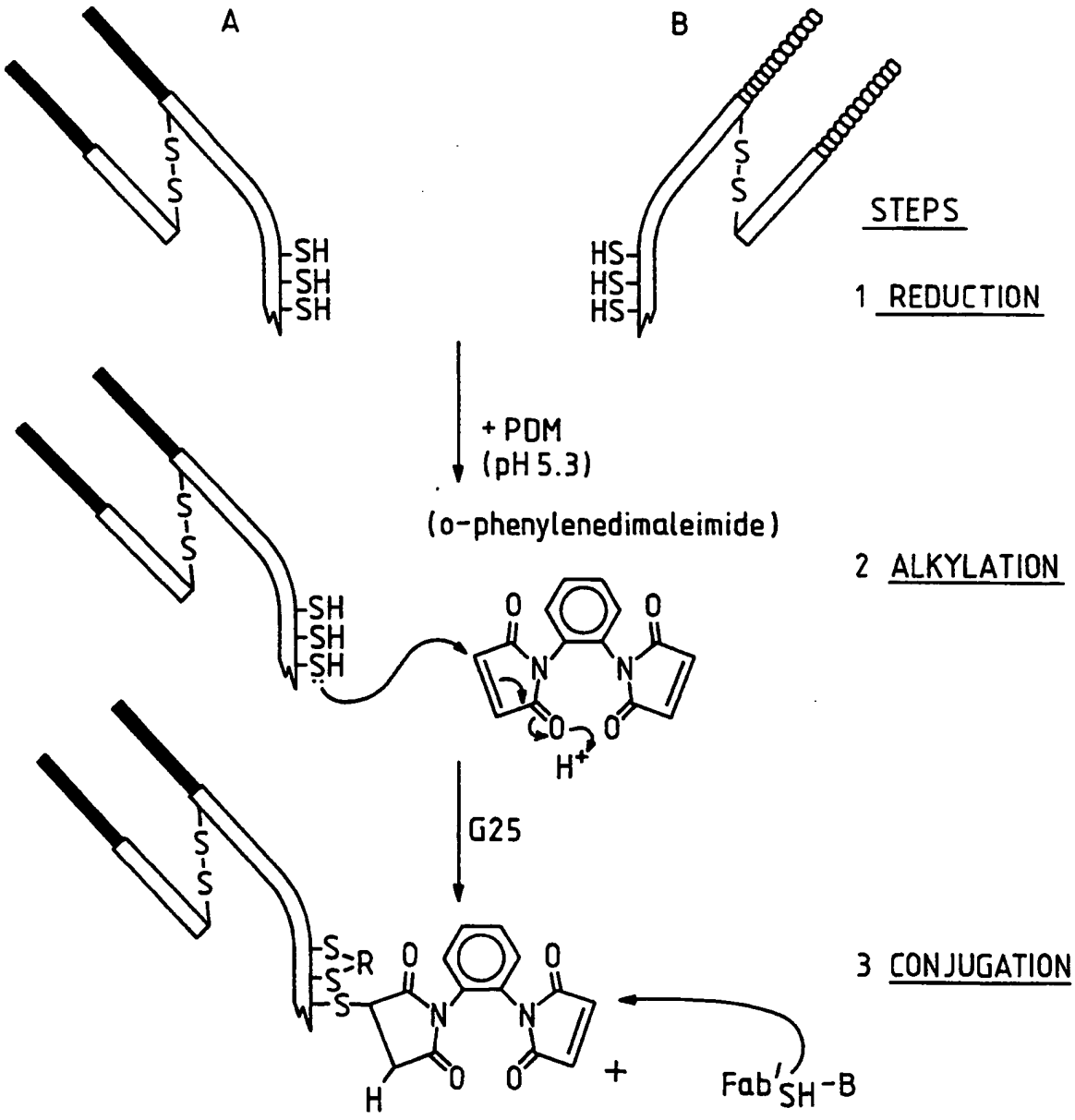
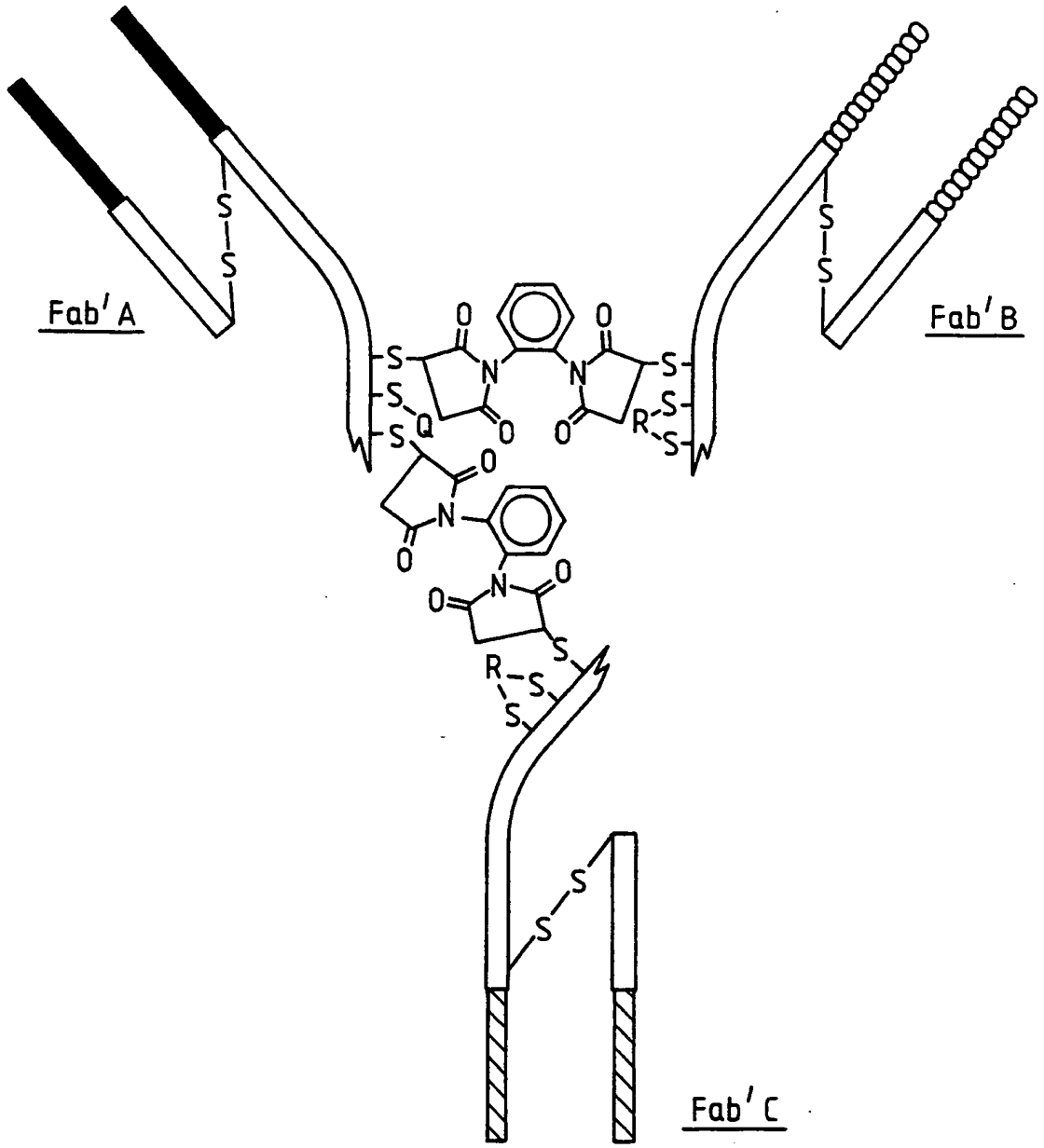

A
B
STEPS
1 REDUCTION
+ PDM
(pH 5.3)
(o-phenylenedimaleimide)
2 ALKYLATION
G25
3 CONJUGATION
Fab'SH-B

Fig. 2.



TRISPECIFIC $F(ab')_3$ ABC

Fig. 3.

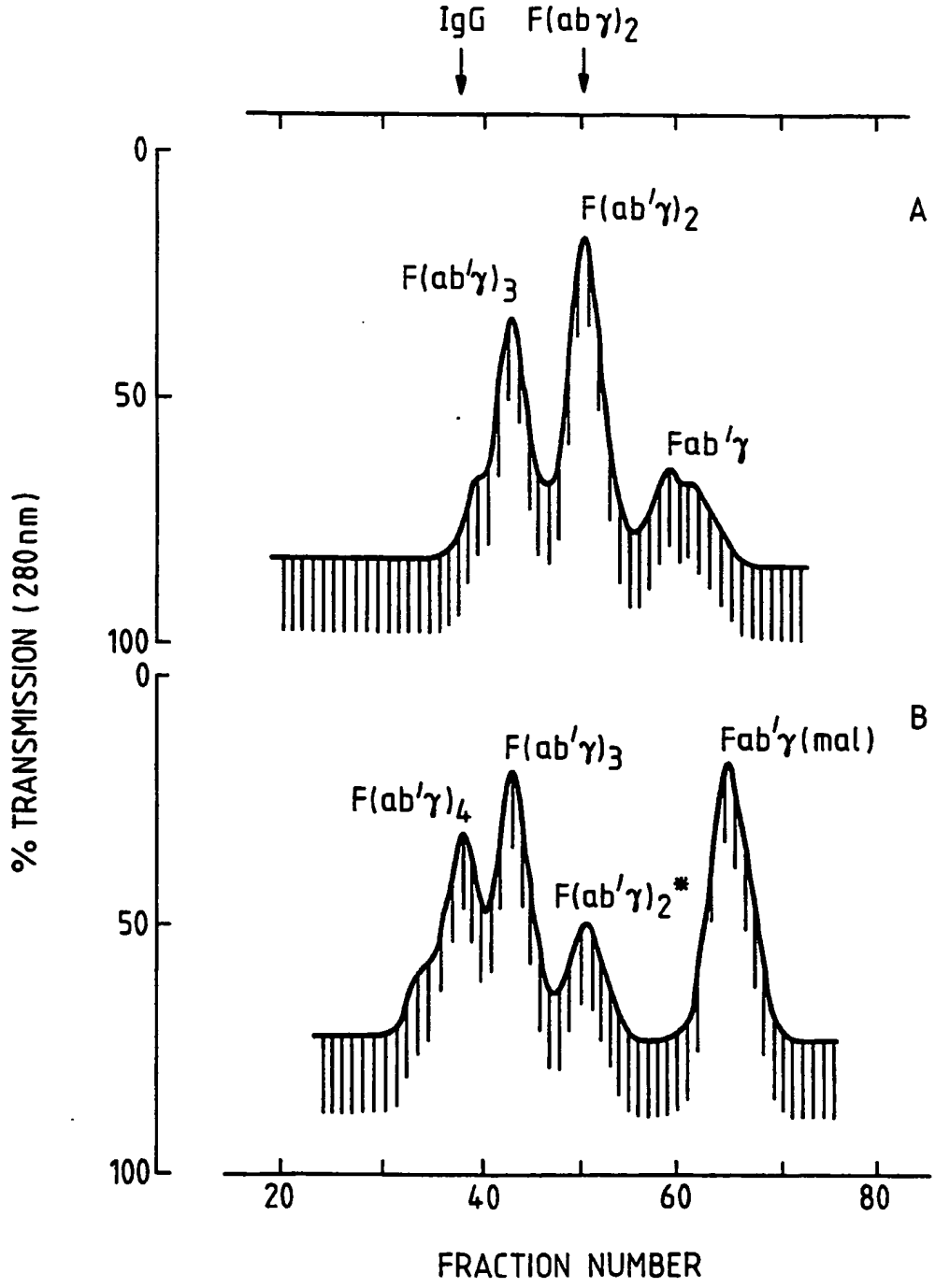

IgG
F(abγ)2
0
50
100
F(ab'γ)2
A
F(ab'γ)3
Fab'γ
0
50
100
B
F(ab'γ)3
Fab'γ(mal)
F(ab'γ)4
F(ab'γ)2*
% TRANSMISSION (280nm)
20
40
60
80
FRACTION NUMBER

Fig. 4.

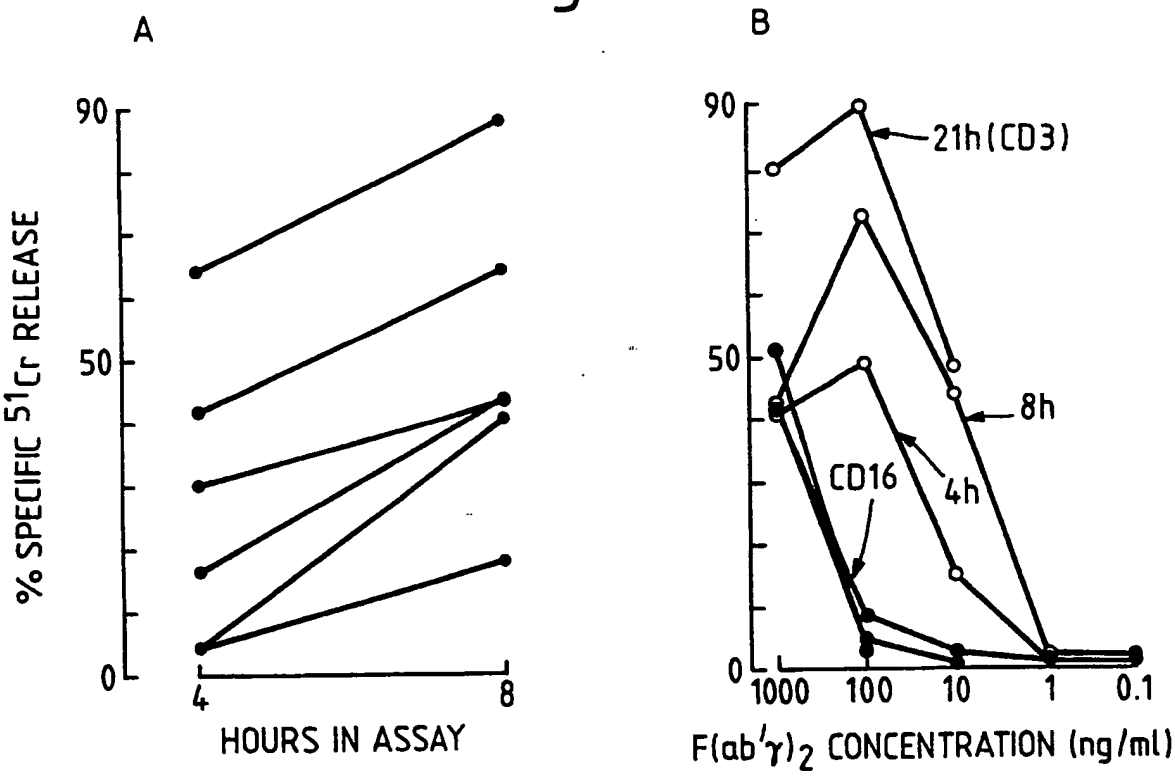

A
90
50
0
% SPECIFIC ^{51}Cr RELEASE
4
8
HOURS IN ASSAY
B
90
50
0
21h(CD3)
8h
4h
CD16
1000
100
10
1
0.1
F(ab'γ)2 CONCENTRATION (ng/ml)

Fig.5.

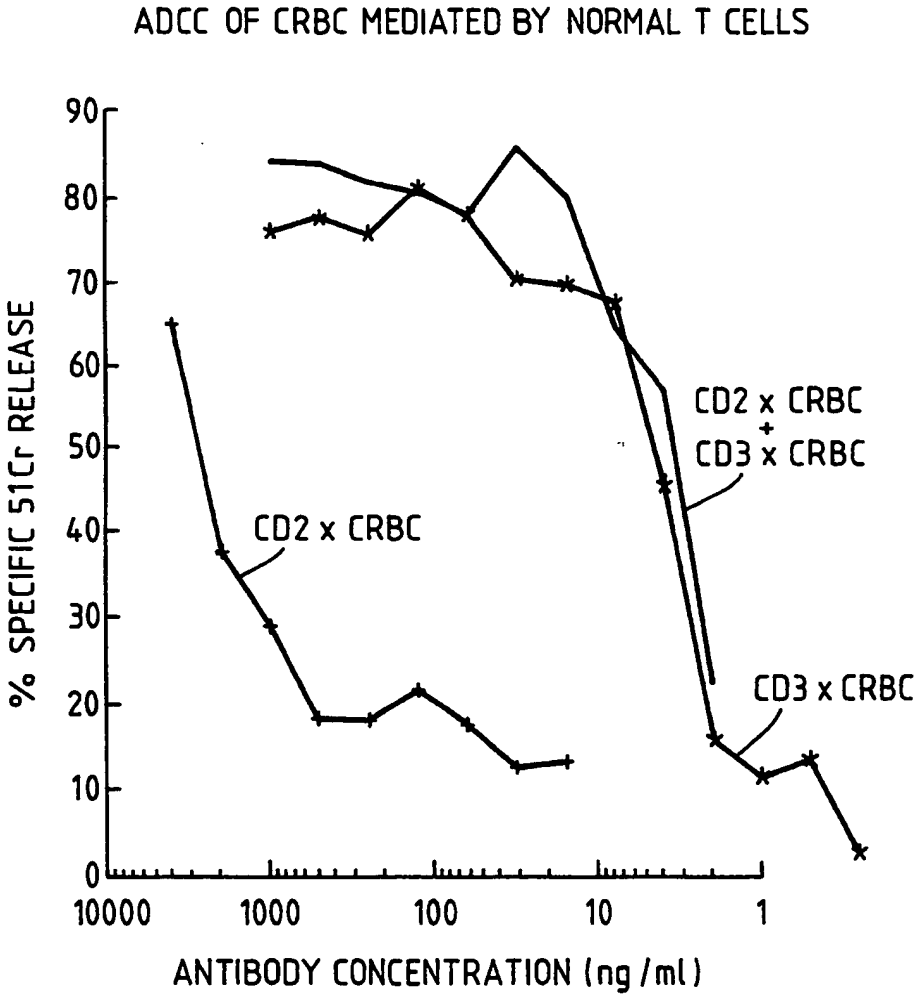

ADCC OF CRBC MEDIATED BY NORMAL T CELLS
% SPECIFIC 51Cr RELEASE
90
80
70
60
50
40
30
20
10
0
10000
1000
100
10
1
ANTIBODY CONCENTRATION (ng/ml)
CD2 x CRBC
+
CD3 x CRBC
CD2 x CRBC
CD3 x CRBC

Fig. 6.

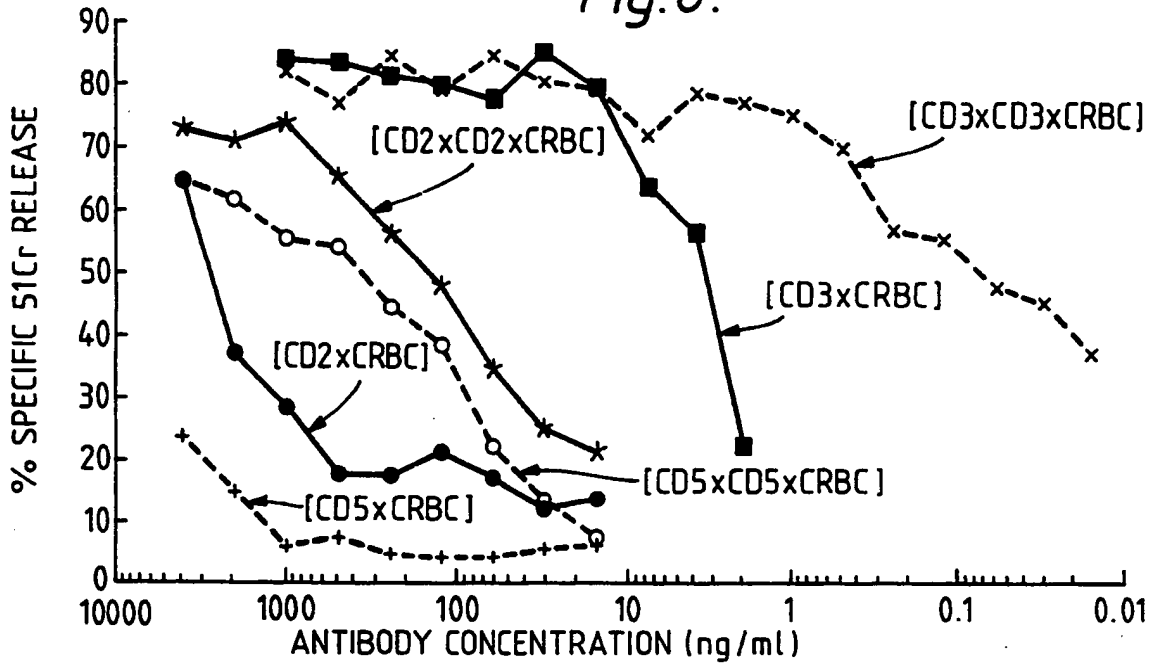

% SPECIFIC 51Cr RELEASE
90
80
70
60
50
40
30
20
10
0
10000
1000
100
10
1
0.1
0.01
ANTIBODY CONCENTRATION (ng/ml)
[CD2xCD2xCRBC]
[CD3xCD3xCRBC]
[CD3xCRBC]
[CD2xCRBC]
[CD5xCD5xCRBC]
[CD5xCRBC]

Fig. 7.

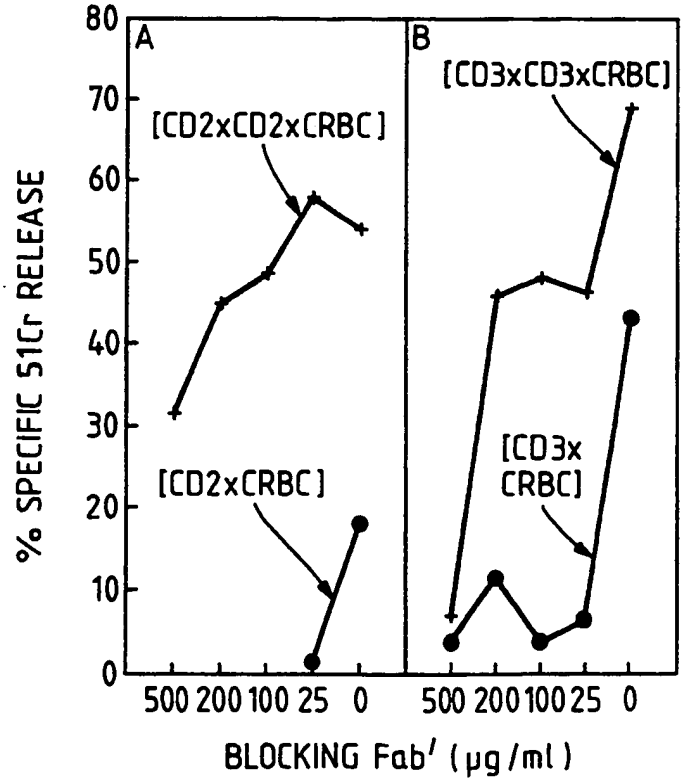

% SPECIFIC 51Cr RELEASE
80
70
60
50
40
30
20
10
0
A
B
[CD2xCD2xCRBC]
[CD2xCRBC]
[CD3xCD3xCRBC]
[CD3x
CRBC]
500 200 100 25 0
500 200 100 25 0
BLOCKING Fab' (µg/ml)

Fig.8.

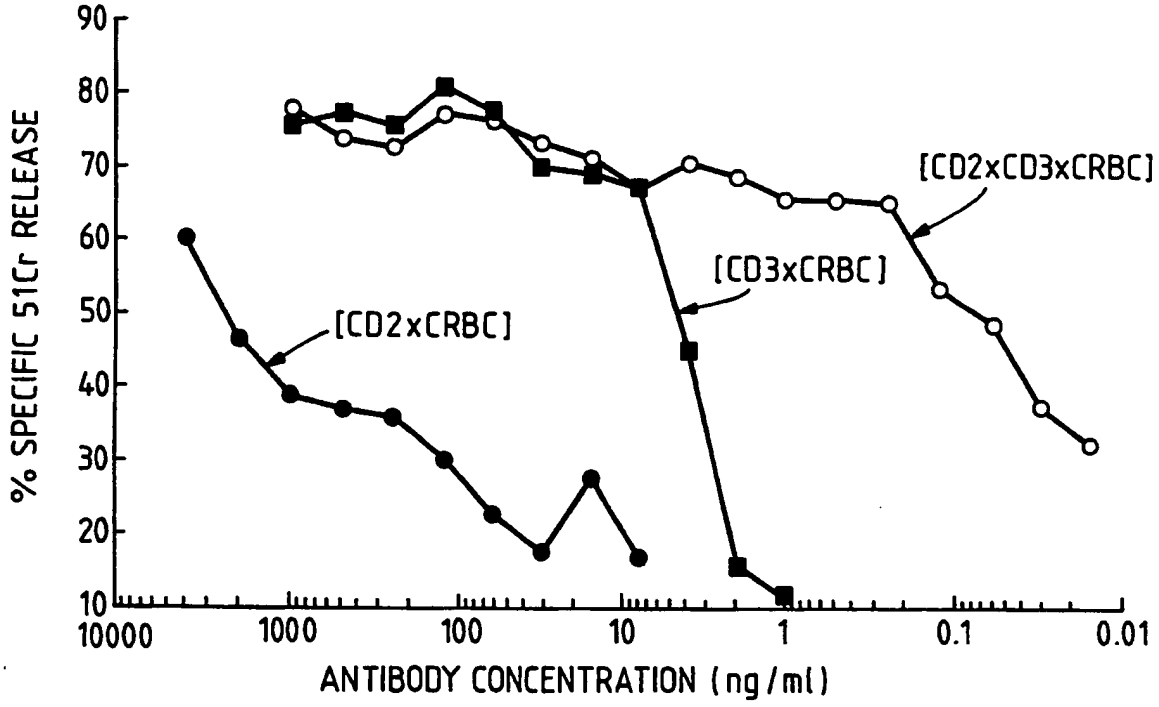

% SPECIFIC 51Cr RELEASE
90
80
70
60
50
40
30
20
10
10000
1000
100
10
1
0.1
0.01
ANTIBODY CONCENTRATION (ng/ml)
[CD2xCD3xCRBC]
[CD3xCRBC]
[CD2xCRBC]

Fig.9.

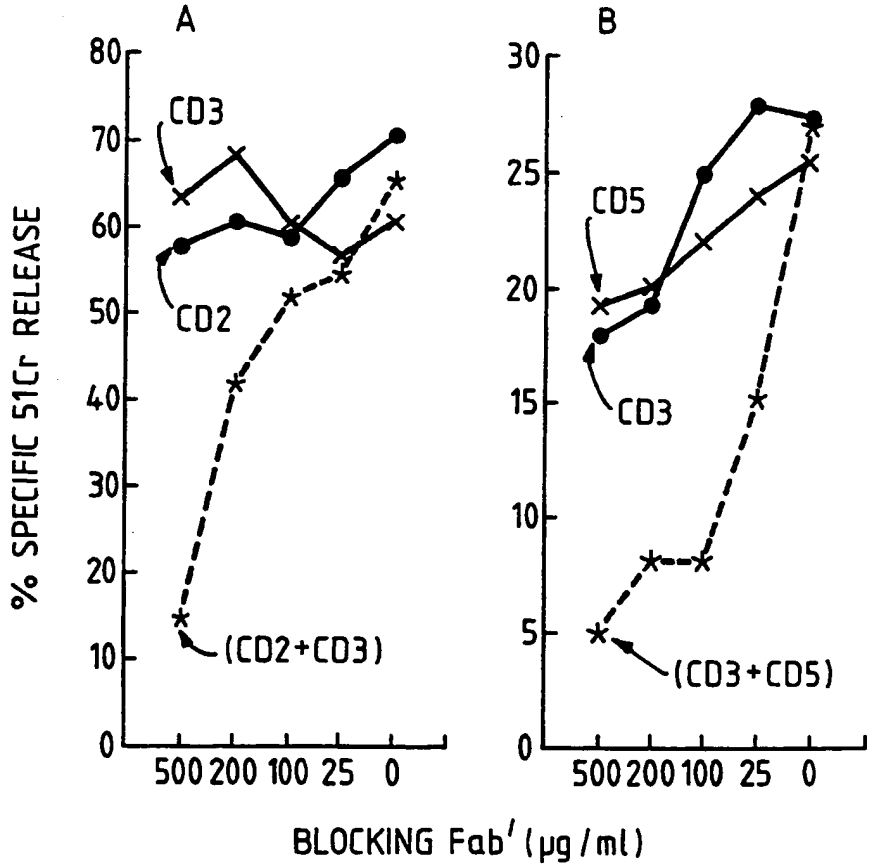
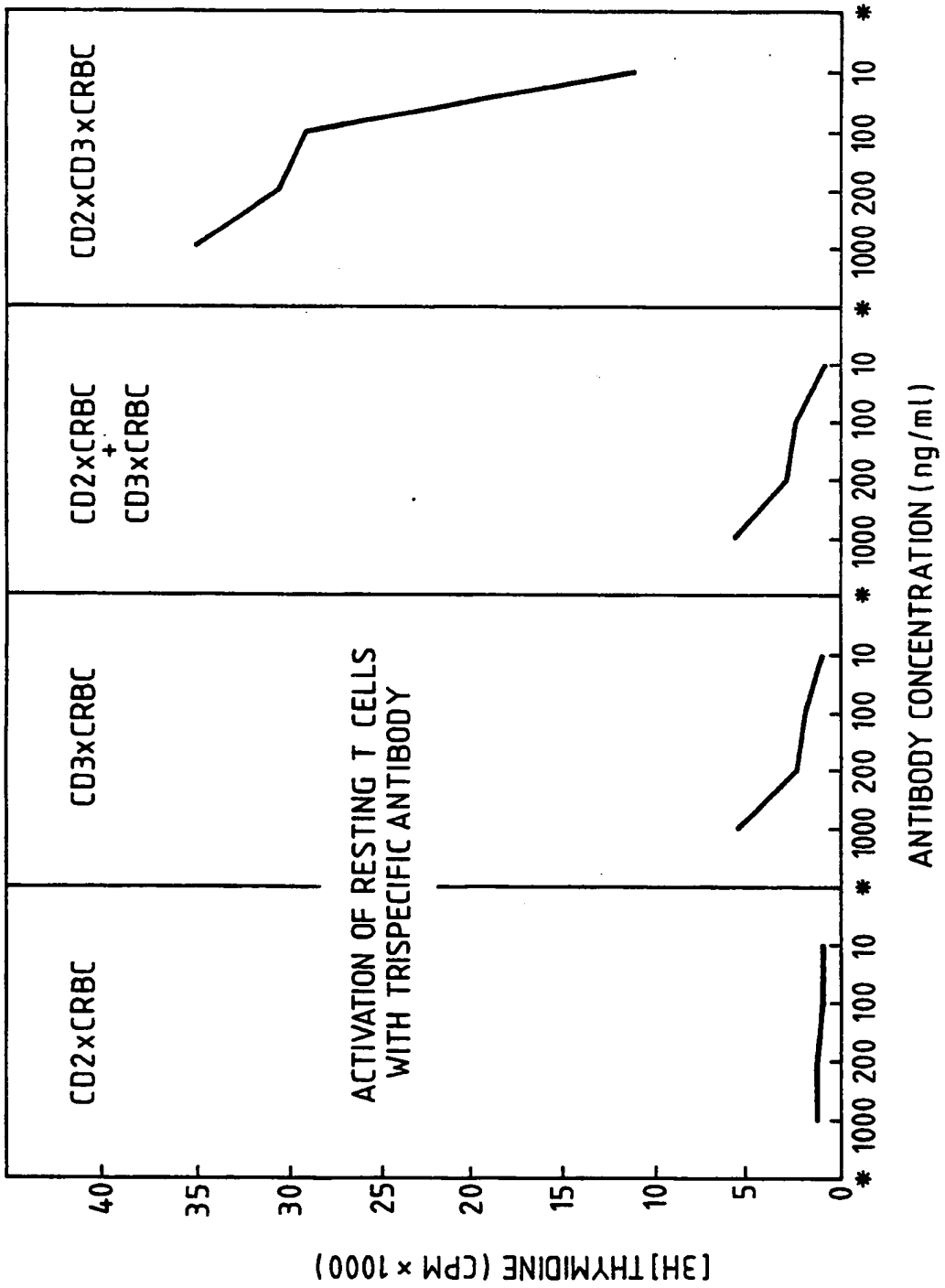

A
B
% SPECIFIC 51Cr RELEASE
CD3
CD2
(CD2+CD3)
CD5
CD3
(CD3+CD5)
80
70
60
50
40
30
20
10
0
30
25
20
15
10
5
0
500 200 100 25 0
500 200 100 25 0
BLOCKING Fab' (µg/ml)

Fig. 10.


CD2xCRBC
CD3xCRBC
CD2xCRBC + CD3xCRBC
CD2xCD3xCRBC
ACTIVATION OF RESTING T CELLS WITH TRISPECIFIC ANTIBODY
[3H] THYMIDINE (CPM x 1000)
0
5
10
15
20
25
30
35
40
* 1000 200 100 10 *
ANTIBODY CONCENTRATION (ng/ml)

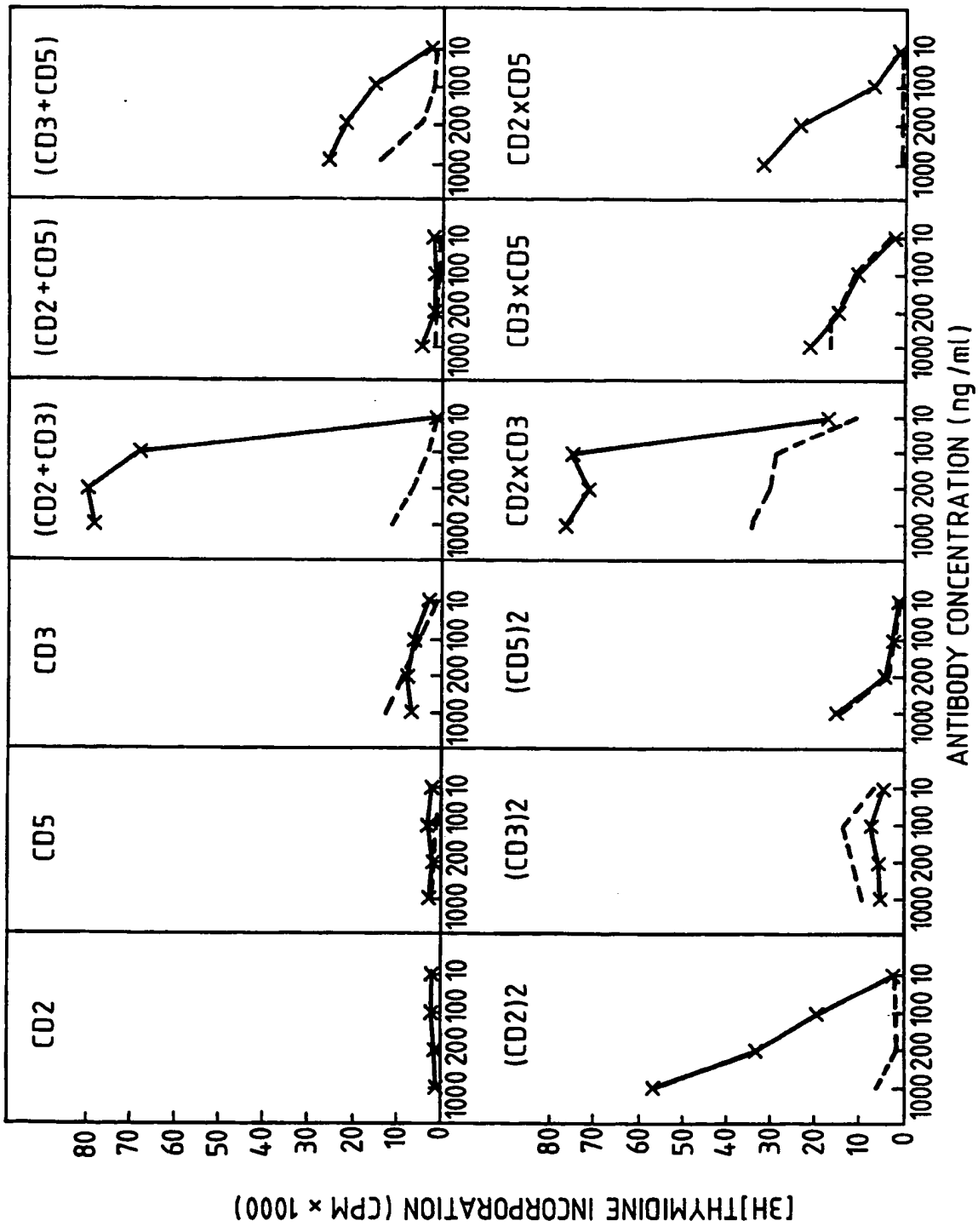

CD2
CD5
CD3
(CD2+CD3)
(CD2+CD5)
(CD3+CD5)
(CD2)2
(CD3)2
(CD5)2
CD2xCD3
CD3xCD5
CD2xCD5
0
10
20
30
40
50
60
70
80
1000 200 100 10
[3H]THYMIDINE INCORPORATION (CPM x 1000)
ANTIBODY CONCENTRATION (ng/ml)

Fig. 11.

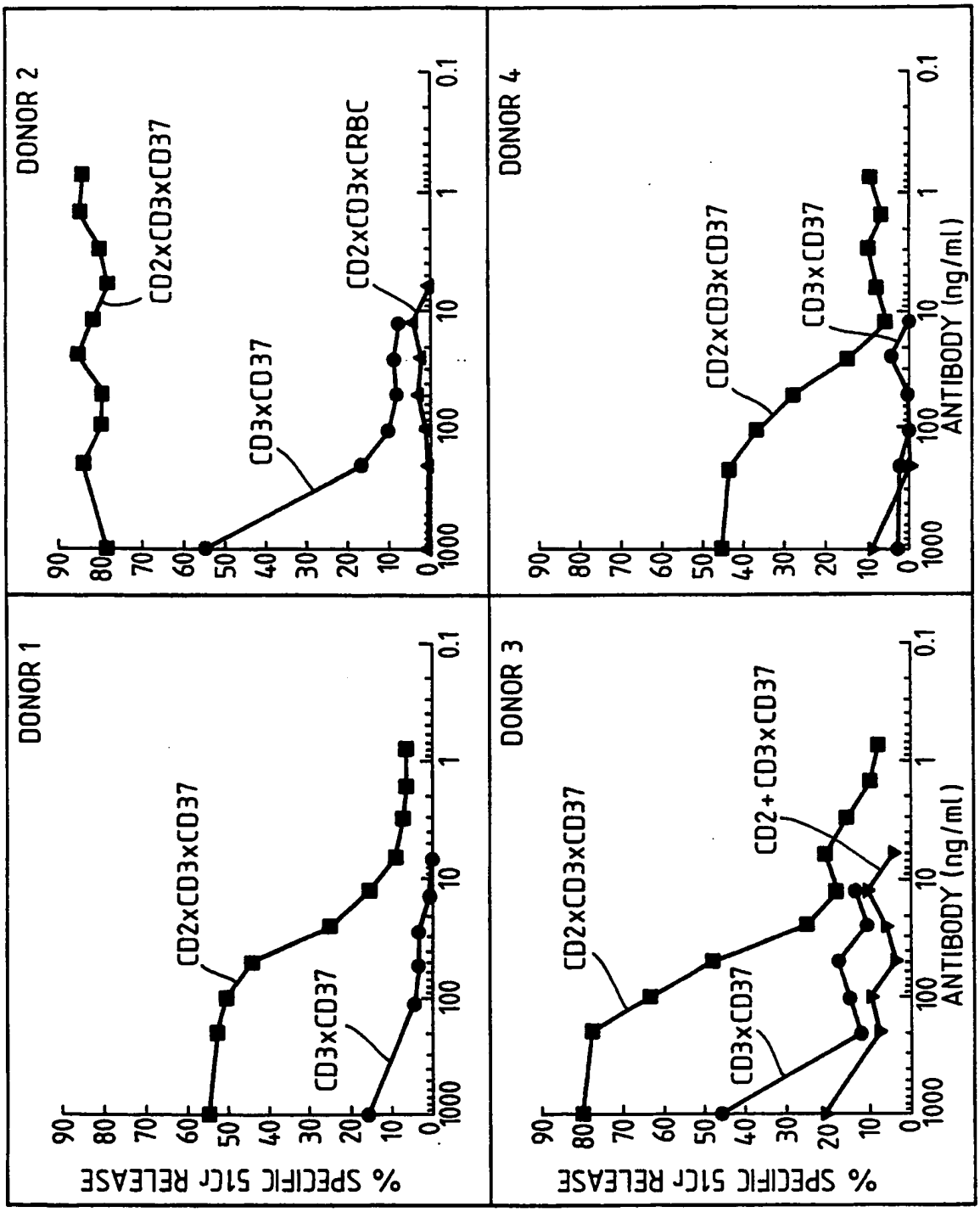
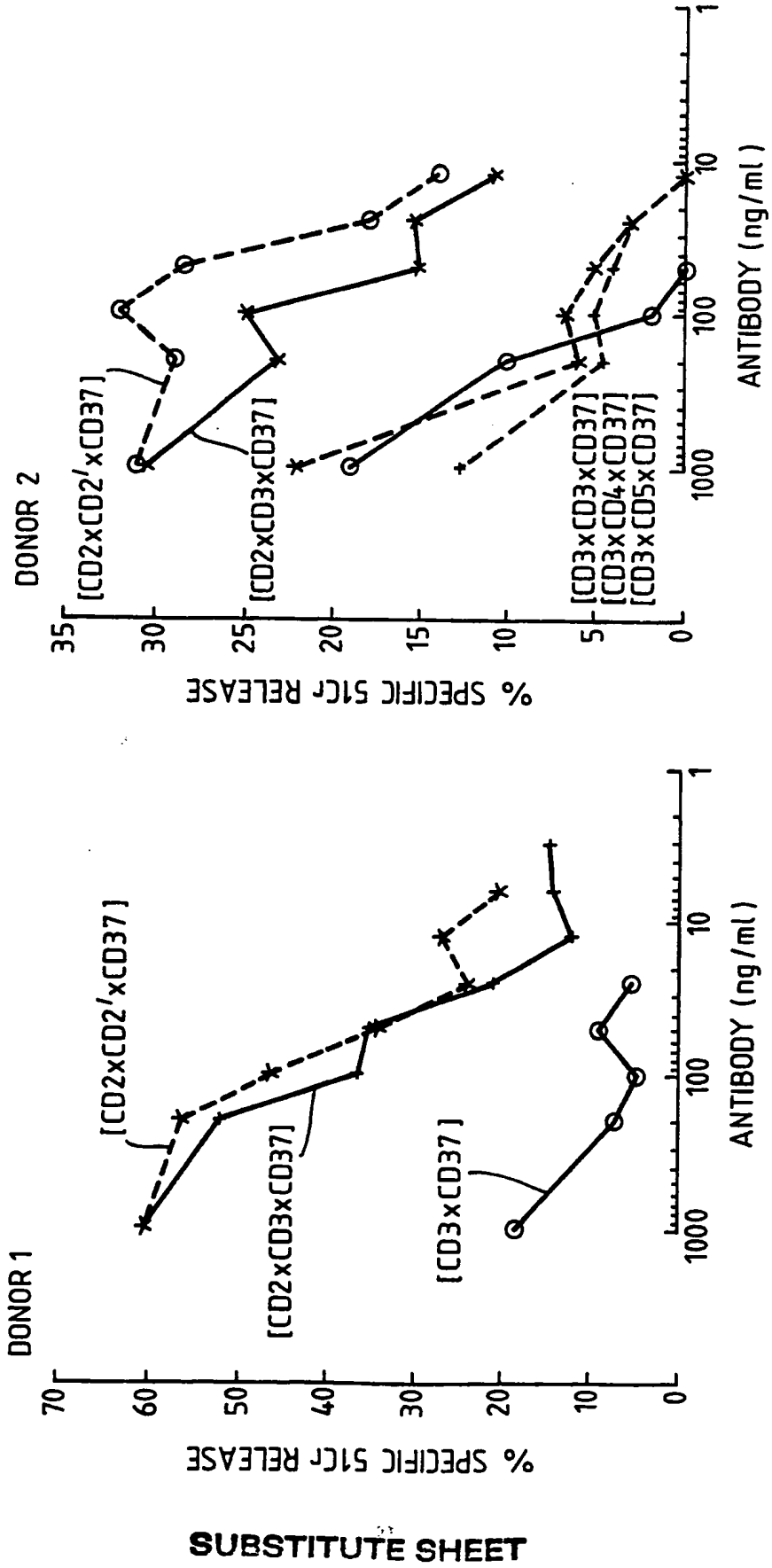

DONOR 1
DONOR 2
DONOR 3
DONOR 4
% SPECIFIC 51Cr RELEASE
ANTIBODY (ng/ml)
CD2xCD3xCD37
CD3xCD37
CD2xCD3xCRBC
CD2+CD3xCD37
0
10
20
30
40
50
60
70
80
90
0.1
1
10
100
1000

Fig. 12.

Fig. 13.


DONOR 1
% SPECIFIC 51Cr RELEASE
70
60
50
40
30
20
10
0
[CD2xCD2'xCD37]
[CD2xCD3xCD37]
[CD3xCD37]
1000
100
10
1
ANTIBODY (ng/ml)
DONOR 2
% SPECIFIC 51Cr RELEASE
35
30
25
20
15
10
5
0
[CD2xCD2'xCD37]
[CD2xCD3xCD37]
[CD3xCD3xCD37]
[CD3xCD4xCD37]
[CD3xCD5xCD37]
1000
100
10
1
ANTIBODY (ng/ml)

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01335

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/28, G 01 N 33/563, H 61 K 39/395, C 12 P21/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO, A1, 9001339 (IMMUNOMEDICS, INC.) 22 February 1990, see page 7 - page 14	1-11,15-32
P,X	EP, A2, 0336379 (ONCOGEN LTD) 11 October 1989, see the whole document	1-7,11,15
P,X	WO, A1, 9004413 (RESEARCH EXPLOITATION LIMITED) 3 May 1990, see page 22 - page 25	23-30
P,X	WO, A1, 8911863 (GLENNIE, MARTIN, JOHN) 14 December 1989, see the whole document	12-14

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30th November 1990	11. 12. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	miss T. MORTENSEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Cancer Detection and Prevention, Vol. 12, 1988, Ian G. Barr et al: "Retargeting of Cytolytic T Lymphocytes by Heteroaggregated (Bispecific) Antibodies ", see page 439 - page 450	1-3
Y		4-32
	--	
X	Chemical Abstracts, volume 107, no. 25, 21 December 1987, (Columbus, Ohio, US), Glennie, Martin J et al: "Preparation and performance of bispecific F (ab'y)2 antibody containing thioether-linked Fab'y fragments ", see page 597, abstract 234429b, & J. Immunol. 1987, 139(7), 2367-2375	1-3
Y		4-32
	--	
X	EP, A2, 0294703 (DANA-FARBER CANCER INSTITUTE, INC.) 14 December 1988, see page 4 - page 10	1-3
Y		4-32
	--	
X	EP, A2, 0241907 (THE GENERAL HOSPITAL CORPORATION) 21 October 1987, see the whole document	1-3
	--	
A	Proc.Natl.Acad.Sci., Vol. 86, May 1989, J M Rojo et al: "Physical association of CD4 and the T-cell receptor can be induced by anti-T-cell receptor antibodies ", see page 3311 - page 3315	4-32
	--	
X	Blood, Vol. 74, No. 2, 1989, Albertus W. Wognum et al: "An Enzyme-Linked Immunosorbent Assay for Erythropoietin Using Monoclonal Antibodies, Tetrameric Immune Complexes, and Substrate Amplification ", see page 622 - page 628	1-3
	--	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Journal of Immunological Methods, Vol. 120, 1989, Terry E. Thomas et al: "Specific binding and release of cells from beads using cleavable tetrameric antibody complexes ", see page 221 - page 231 --	1-3
Y	EP, A2, 0180171 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 7 May 1986, see claims --	1-3,5-7, 31,32

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/01335

SA 39700

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 01/11/90
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9001339	22/02/90	AU-D-	4062889	05/03/90
		EP-A-	0353960	07/02/90
		US-A-	4925648	15/05/90
EP-A2- 0336379	11/10/89	AU-D-	3242389	05/10/89
		JP-A-	1304356	07/12/89
WO-A1- 9004413	03/05/90	NONE		
WO-A1- 8911863	14/12/89	AU-D-	3752089	05/01/90
EP-A2- 0294703	14/12/88	JP-A-	64003128	06/01/89
EP-A2- 0241907	21/10/87	JP-T-	2500321	08/02/90
		WO-A-	87/06240	22/10/87
EP-A2- 0180171	07/05/86	JP-A-	61234779	20/10/86